09/828,211

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☐ 1. 6287822. 04 Aug 98; 11 Sep 01. Mutation detection method. Gjerde; Douglas T, et al. 435/91.2; 210/198.2 210/635 435/6 536/22.1 536/25.4. C12P019/34 C12Q001/68 C07H019/00 C07H021/04 B01D015/08.

☐ 2. 5795976. 08 Aug 95; 18 Aug 98. Detection of nucleic acid heteroduplex molecules by denaturing high-performance liquid chromatography and methods for comparative sequencing. Oefner; Peter Josef, et al. 536/25.4; 204/450 204/456 435/6 435/91.2 536/22.1 536/23.1 536/24.3. C12N015/10 C07H021/02 C12Q001/68 C12P019/34.

☐ 3. 4879214. 15 Nov 88; 07 Nov 89. Differentiation of nucleic acid segments on the basis of nucleotide differences. Kornher; John S., et al. 435/6; 435/91.2. C12Q001/68.

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L3	L2 and heteroduplex$2 and homoduplex$2	6	L3
L2	L1 and (reverse phase near5 chromatograph$)	39	L2
L1	(detect$ or determin$) near5 mismatch	3569	L1

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L4: Entry 3 of 3 File: USPT Nov 7, 1989

DOCUMENT-IDENTIFIER: US 4879214 A
TITLE: Differentiation of nucleic acid segments on the basis of nucleotide differences

Brief Summary Paragraph Right (7):
A technique for detecting specific mutations in any segment of DNA is described in Wallace, et al., Nucl. Acids Res. 9:879-894 (1981). It involves hybridizing the DNA to be analyzed (target DNA) with a complementary, labeled oligonucleotide probe. Due to the thermal instability of DNA duplexes containing even a single base pair mismatch, differential melting temperature can be used to distinguish target DNAs that are perfectly complementary to the probe from target DNAs that differ by as little as a single nucleotide. An adaptation of this technique, described by Saiki, et al., U.S. Pat. No. 4,683,194, can be used to detect the presence or absence of a specific restriction site. In Saiki's adaptation, an end-labeled oligonucleotide probe spanning a restriction site is hybridized to the target DNA. The hybridized duplex of DNA is then appropriately incubated with the restriction enzyme for that site. Only paired duplexes between probe and target that reform the restriction site will be cleaved by digestion with the restriction endonuclease. Detection of shortened probe molecules indicates that the specific restriction site is present in the target DNA. In a related technique, described in Landegren, et al., Science 241:1077-1080 (1988), oligonucleotide probes are constructed in pairs such that their junction corresponds to the site on the DNA being analyzed for mutation. These oligonucleotides are then hybridized to the DNA being analyzed. Base pair mismatch between either oligonucleotide and the target DNA at the junction location prevents the efficient joining of the two oligonucleotide probes by DNA ligase. A major problem with these and other oligonucleotide techniques is that the mutation must already be characterized as to type and location in order to synthesize the proper probe. Thus, techniques using oligonucleotide probes can be used to assay for specific, known mutations, but they cannot be used generally to identify previously undetected mutations.

Brief Summary Paragraph Right (8):
In the technique described in Mundy, U.S. Pat. No. 4,656,127, specific mutations can be detected by first hybridizing a labeled DNA probe to the target nucleic acid in order to form a hybrid in which the 3' end of the probe is positioned adjacent to the specific base being analyzed. Then, a DNA polymerase is used to add a nucleotide analog, such as a thionucleotide, to the probe strand, but only if the analog is complementary to the specific base being analyzed. Finally, the probe-target hybrid is treated with exonuclease III. If the nucleotide analog has been incorporated, the labeled probe is protected from nuclease digestion. Absence of a labeled probe indicates that the analog and the specific base being analyzed were not complementary. As with abovediscussed techniques involving oligonucleotides, this method detects specific mutations, but it cannot be used in a general manner to detect all possible nucleotide differences.

Brief Summary Paragraph Right (9):
Nucleotide differences between two DNA sequences also can be studied by forming a heteroduplex between the two DNAs of interest. Base pair mismatches will occur within the heteroduplex at points where the sequences differ. A number of methods have been developed to detect such mismatches. Chemical probes for mismatches exist that specifically react with those atoms in the base normally involved in hydrogen bonding, see, e.g., Novack, et al., Proc. Natl. Acad. Sci. U.S.A. 83:586-590 (1986); Cotton, et al., Proc. Natl. Acad. Sci. U.S.A. 85:4397-4401 (1988). These chemically altered sites are susceptible to chemical cleavage, whereas a perfectly paired duplex is not. Problems with this technique include: (i) toxicity of the chemical reagents and (ii)

efficiency of much less than 100% for the reactions with unpaired bases. Another approach to mismatch detection is based upon the ability of certain nucleases to recognize and cleave these sites. S.sub.1 nuclease and RNase A have been shown effective in mismatch detection and cleavage, see, e.g., Shenk, et al., Proc. Natl. Acad. Sci. U.S.A. 72:989-993 (1975); Myers, et al., Science 230:1242-1246 (1985). Neither of these enzymes, however, cleaves at all possible mismatched base pairs. There is also considerable background associated with nuclease cleavage at perfectly paired sites in the duplex.

Brief Summary Paragraph Right (10):
Myers, et al., Nature 313:495-498 (1985), and Fischer, et al., Proc. Natl. Acad. Sci. U.S.A. 80:1579-1583 (1983), have demonstrated that mismatched base pairs within a heteroduplex alter its melting properties with respect to a perfectly paired homoduplex. These altered melting properties can be observed electrophoretically on a gel containing an exponential gradient of denaturant. A serious drawback to this technique is the difficulty of manipulating and processing these gels. Another problem is the inability of this technique to detect uniformly all base pair mismatches along a given heteroduplex. The resolving power of these gels is reduced with increasing GC content of the heteroduplex, and thus mutations in a GC-rich domain are more difficult to detect than mutations in a domain with a lower GC content.

Brief Summary Paragraph Right (11):
The primer extension process described in Proudfoot, et al., Science 209:1329-1336 (1980), has been widely used to study the structure of RNA and also has been used to characterize DNA, see, e.g. Engelke, et al., Proc. Natl. Acad. Sci. U.S.A. 85:544-548 (1988). This process consists of hybridizing a labeled oligonucleotide primer to a template RNA or DNA and then using a DNA polymerase and deoxynucleoside triphosphates to extend the primer to the 5' end of the template. The labeled primer extension product is then fractionated on the basis of size, usually by electrophoresis through a denaturing polyacrylamide gel. When used to compare homologous DNA segments, this process can detect differences due to nucleotide insertion or deletion. Because size is the sole criterion used to characterize the primer extension product, this method cannot detect differences due to nucleotide substitution.

Brief Summary Paragraph Right (39):
In order to compare the mobility of the synthesized strands, the strands must be detected. Any method used to detect nucleic acid can be used to detect the synthesized strands containing mobility-shifting nucleotide analogs. One method is to perform the primer extension reaction with a labeled primer. Typically, oligonucleotide primers are labeled by attaching a reporter to the 5' end of the oligonucleotide. This reporter can be a radioactive isotope; a group that takes part in an enzyme or fluorescent or chemiluminescent reaction; or some other small molecule, such as biotin, that can be detected by a convenient method. Alternatively, labeling can be performed by incorporating reporter-tagged nucleotides into the synthesized strands as they are synthesized. The reporter can be attached either to a mobility-shifting nucleotide analog or to one of the other nucleotides used as substrates in the preparation of synthesized strands. The same type of reporters used to label primers can be used to label the nucleotide substrates. Another method of detection entails hybridizing a reporter-labeled probe to the synthesized strands after the size fractionation step, as in the Southern blot procedure.

Detailed Description Paragraph Right (6):
Next, the triethylammonium salt of
5-(3-biotinamido(hexanoylamido))-1-propynyl)-2'-deoxycytidine 5'-triphosphate, 5-(Bio-AC-AP3)dCTP, ##STR5## was prepared from 5-(3-amino-1-propynyl)-2'-deoxycytidine 5'-triphosphate, 5-(AP3)dCTP. 5-(3-Amino-1-propynyl)-2'-deoxycytidine 5'-triphosphate (30 umol) was dissolved in 1 M aqueous TEAB, pH 7.6, (600 .mu.L) and sulfosuccinimidyl 6-(biotinamido)-hexanoate Na salt (Piece Chemical Co., Rockford, IL; 33 mg; 60 .mu.mol) was added. The solution was held at 50.degree. C. for 90 min and then diluted to 6 mL with water. The solution was loaded onto a DEAE Sephadex A-25-120 column (1.times.19 cm) that had been equilibrated with 1.0 M aqueous TEAB, pH 7.6. The column was eluted with a linear gradient of TEAB, pH 7.6, from 0.1 M (150 mL) to 1.0 M (150 mL), running at c. 100 mL/hr, collecting fractions every 3 min. The eluent was monitored by absorbance at 270 nm, and the fractions corresponding to the second peak (#22-33) were pooled, stripped, and co-evaporated (2x) with ethanol. The residue was

subjected to reverse phase chromatography (Baker 7025-00) on a column (1.times.8 cm) poured in acetonitrile/1 M aqueous TEAB, pH 7.6. The column was eluted with a step gradient of acetonitrile/1 M TEAB, pH 7.6, (0-30% acetonitrile, 2%/step, 2 mL/step, 1 fraction/step). The fractions were assayed by HPLC on a reverse phase column under conditions analogous to those used in the above separation. Starting material eluted first (#2-6) followed by the product (#7-11). The product fractions were pooled, stripped, and coevaporated (2x) with ethanol. The yield, assuming an absorption coefficient of 10,100 at 295.5 nm, was 10.5 umol (35%). HPLC on Zorbax SAX and on reverse phase showed the material to be >99% pure. The .sup.1 H- and .sup.31 P-NMR spectra are fully consistent with the title structure.

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L4: Entry 2 of 3 File: USPT Aug 18, 1998

DOCUMENT-IDENTIFIER: US 5795976 A
TITLE: Detection of nucleic acid heteroduplex molecules by denaturing high-performance liquid chromatography and methods for comparative sequencing

Abstract Paragraph Left (1):
The present invention describes a method for separating heteroduplex and homoduplex DNA molecules in a mixture. In the method, such a mixture is applied to a stationary reverse phase support. The heteroduplex and homoduplex molecules are eluted with a mobile phase containing an ion-pairing reagent and an organic solvent. The eluting is carried out under conditions effective to at least partially denature the heteroduplexes (e.g., thermal or chemical denaturing) resulting in the separation of the heteroduplexes from the homoduplexes. The method has many applications including, but not limited to, comparative nucleic acid sequencing, linkage analysis, evolutionary studies, forensics, identification of disease-causing gene mutations, genetic marker development and diagnostics.

Brief Summary Paragraph Right (1):
The present invention relates to a chromatographic method for detecting heteroduplexes in nucleic acid fragments, and particularly to denaturing high performance liquid chromatography, for use in detecting mutations and for comparative DNA sequencing.

Brief Summary Paragraph Right (11):
Presently used methods for screening for polymorphic sites within a gene include RNase A cleavage, chemical cleavage, denaturing gradient gel electrophoresis. These methods exploit characteristics of mismatched heteroduplexes formed between normal and mutant sequences.

Brief Summary Paragraph Right (14):
In denaturing gradient gel electrophoresis (DGGE), either homoduplex or heteroduplex double stranded DNA is electrophoresed under denaturing conditions of increasing concentration until the last domain is denatured, and migration of the DNA through the gel is retarded. DNA sequences differing by a single base pair migrate at different rates along the gel, thereby allowing detection of a polymorphic site, if present.

Brief Summary Paragraph Right (17):
The ligase-mediated method for detecting mutations makes use of the fact that the ends of two single strands of DNA must be exactly aligned for DNA ligase to join them. In utilizing this technique, oligonucleotides complementary to the target sequence, 5' to and including the mutation site, are synthesized and labeled. A third oligonucleotide complementary to the common sequence 3' to the mutation site is synthesized and also labeled. The oligonucleotides are then hybridized to strands of the target. In cases in which the 5' and 3' oligonucleotides form a flush junction that can be joined by DNA ligase, ligation occurs. However, a single base pair mismatch occurring between the normal 5' oligonucleotide and the mutation site is sufficient to prevent the ligase from acting and can readily be detected.

Brief Summary Paragraph Right (19):
The present invention provides a method for separating heteroduplex and homoduplex nucleic acid molecules (e.g., DNA or RNA) in a mixture using high performance liquid chromatography. In the separation method, a mixture containing both heteroduplex and homoduplex nucleic acid molecules is applied to a stationary reverse-phase support. The sample mixture is then eluted with a mobile phase containing an ion-pairing reagent and an organic solvent. Sample elution is carried out under conditions effective to at least partially denature the heteroduplexes and results in the separation of the heteroduplex and homoduplex molecules.

Brief Summary Paragraph Right (23):
At least partial denaturation of heteroduplex molecules can be carried out several ways including the following. Temperatures for carrying out the separation method of the invention are typically between about 50.degree. and 70.degree. C., preferably between about 55.degree.-65.degree. C. In a preferred embodiment, the separation is carried out at 56.degree. C. Alternatively, in carrying out a separation of GC-rich heteroduplex and homoduplex molecules, a higher temperature (e.g., 64.degree. C.) is preferred.

Brief Summary Paragraph Right (24):
Alternately, sample elution is carried out under pH conditions effective to at least partially denature the heteroduplex molecules. In such cases, a lower column temperature less than about 65.degree. C. may be sufficient for the separation of the heteroduplex and homoduplexes molecules in the sample.

Brief Summary Paragraph Right (26):
In one particular embodiment of the present method, homoduplex and heteroduplex molecules in a mixture are separated by applying the mixture to a C-18 alkylated polystyrene-divinylbenzene copolymer stationary support and eluting the mixture with a mobile phase containing triethylammonium acetate as the ion-pairing reagent and acetonitrile as the organic solvent at a temperature between about 50.degree.-65.degree. C.

Brief Summary Paragraph Right (27):
In an alternate embodiment, the homoduplex and heteroduplex molecules contained in the mixture are amplified using the polymerase chain reaction and the amplified DNA molecules are denatured and renatured to form a mixture of heteroduplex and homoduplex molecules prior to carrying out the separation method of the invention.

Brief Summary Paragraph Right (30):
Also disclosed is a method for comparative DNA sequencing in which potentially all possible nucleotide mismatches and insertion/deletions within select amplified DNA fragments obtained from multiple animal or human subjects can be detected. In the context of the present invention, comparative DNA sequencing is carried out by amplifying DNA samples, typically up to at least about 1.5 kb in length, obtained from multiple subjects. The amplified DNA fragments are then surveyed, either individually or in pools containing up to about 10 samples, for the presence or absence of heteroduplexes using the denaturing high performance liquid chromatography method of the present invention.

Brief Summary Paragraph Right (31):
In surveying the samples, the amplified DNA fragments are denatured and allowed to reanneal. The resulting mixture of DNA fragments is then applied to a stationary reverse-phase support. The sample mixture is eluted with a mobile phase containing an ion-pairing reagent and an organic solvent. Sample elution is carried out under conditions effective to at least partially denature any heteroduplexes present in the sample and results in the detection of any heteroduplex molecules contained in the sample. The detection of a heteroduplex indicates the presence of a base pair mismatch and/or an insertion/deletion in the sample fragment(s).

Brief Summary Paragraph Right (32):
In instances in which only homoduplexes are observed during the sample screening, further standard sequencing is not required since the sequence is monomorphic (i.e., lacking a polymorphic site) in all subjects compared. In utilizing the method of the present invention, only those DNA fragments identified as heteroduplexes, and therefore identified as containing at least one polymorphic site, are then sequenced by conventional methods to characterize the observed polymorphism(s).

Drawing Description Paragraph Right (5):
FIG. 5 is a scheme illustrating the mixing of two different double-stranded DNA fragments, subsequent denaturing, and reannealing to produce both homoduplex (2 species) and heteroduplex (2 species) products;

Drawing Description Paragraph Right (6):
FIGS. 6A-6C are IP-RP-HPLC chromatograms illustrating the detection at 50.degree. C.

of a 209-base pair homoduplex "homo-A-209" (FIG. 6A), a 209-base pair homoduplex "homo-G-209" (FIG. 6B), and a chromatogram of the hybrids formed after mixing homoduplexes A and G, denaturing, and reannealing to produce both homoduplex and heteroduplex products (FIG. 6C);

Drawing Description Paragraph Right (8):
FIGS. 8A-8C are IP-RP-HPLC chromatograms illustrating the detection (at 56.degree. C.) of two 439 base pair heteroduplexes each containing a single site mismatch in a sample containing both 439-mer homoduplex and heteroduplex samples (FIG. 8C). FIGS. 8A and 8B are chromatograms of each of the two different 439-mer homoduplexes designated as "homo-A-439" and "homo-G-439", respectively, prior to mixing, denaturing, and reannealing;

Drawing Description Paragraph Right (9):
FIG. 9A-9C are IP-RP-HPLC chromatograms of each of two PCR-amplified 1-kilobase homoduplexes, "homo-A-1kb" (FIG. 9A) and "homo-G-1kb" (FIG. 9B). FIG. 9C illustrates the detection, at 56.degree. C., of two 1-kilobase heteroduplexes, each containing a single base-pair mismatch, in a mixture also containing two 1-kilobase homoduplexes;

Drawing Description Paragraph Right (10):
FIG. 10-10C are IP-RP-HPLC chromatograms illustrating the separation of 209-base pair homoduplexes from 209-base pair heteroduplexes as a function of column temperature;

Drawing Description Paragraph Right (12):
FIGS. 12A-C are IP-RP-HPLC chromatograms of each of two PCR-amplified 1.5-kilobase homoduplexes (FIGS. 12A and 12B) and the detection, at pH 7.5, of two 1.5-kilobase heteroduplexes, each containing a single base-pair mismatch (FIG. 12C); and

Drawing Description Paragraph Right (13):
FIGS. 13A-13E are IP-RP-HPLC chromatograms of a segment of the .beta.-globin gene containing a polymorphic site flanked by a GC-rich region, illustrating the effect of temperature on sample resolution for detecting heteroduplexes.

Detailed Description Paragraph Right (3):
"Ion-pair (IP) chromatography" as used herein refers to any chromatographic method for separating samples in which some or all of the sample components contain functional groups which are ionized or are ionizable. Ion-pair chromatography is typically carried out with a reverse phase column in the presence of an ion-pairing reagent.

Detailed Description Paragraph Right (7):
"Ion pairing reverse phase high performance liquid chromatography (IP-RP-HPLC)" refers to a type of high performance liquid chromatography in which the solid support is a reverse phase support and the mobile phase contains an ion-pairing reagent.

Detailed Description Paragraph Right (10):
"Homoduplex molecules" are typically composed of two complementary DNA strands.

Detailed Description Paragraph Right (11):
"Heteroduplex molecules" are typically composed of two complementary nucleic acid strands (e.g., DNA or RNA), where the strands have less than 100% sequence complementarity. The functional definition of homoduplex and heteroduplex molecules, in the context of the present invention, is apparent from the results presented below. Typically, in a mixed population of homoduplex and heteroduplex molecules, for shorter strands (e.g., typically about less than 70 base pairs in size) heteroduplex molecules elute as peaks corresponding to their respective denatured single strands under select denaturing conditions using reverse phase ion pairing HPLC, separable from those of homoduplex molecules. In a mixed population of homoduplex and heteroduplex molecules larger than about 70 base pairs in length, heteroduplex molecules typically elute with shorter retention times than those of homoduplexes of essentially the same size under select denaturing conditions using reverse phase ion pairing HPLC. "Insertions or deletions" or "indels" can occur in duplexes consisting of two complementary DNA strands, where the first strand of the DNA contains a greater number of nucleotides at an internal site than the second strand DNA molecule, and where these extra nucleotides are flanked by paired-complementary sequences. Indels can occur in heteroduplexes.

Detailed Description Paragraph Right (12):
"Base-pair mismatches" typically refers to a single base-pair mismatch flanked by matched base-pairs. Base-pair mismatches also include a series of mismatched base-pairs flanked by matched base-pairs. Base-pair mismatches can occur in heteroduplexes.

Detailed Description Paragraph Right (13):
A heteroduplex molecule that is "at least partially denatured" under a given set of chromatographic conditions refers to a molecule in which several complementary base pairs of the duplex are not hydrogen-bond paired, such denaturing typically extending beyond the site of the base-pair mismatch contained in the heteroduplex, thereby enabling the heteroduplex to be distinguishable from a homoduplex molecule of essentially the same size. In accordance with the present invention, such denaturing conditions may be either chemically (e.g., resulting from pH conditions) or temperature-induced, or may be the result of both chemical and temperature factors.

Detailed Description Paragraph Right (14):
"Comparative DNA sequencing" as used herein refers to a method for detecting any, and preferentially all, possible nucleotide mismatches and insertion/deletions within select amplified or non-amplified DNA fragments obtained from multiple animal or human subjects. In comparative DNA sequencing, DNA samples, typically up to at least about 1.5 kb in length, are obtained from multiple subjects and amplified or are otherwise produced (e.g., by cloning). The amplified DNA fragments are then surveyed, either individually or in pools containing up to about 10 unique samples, for the presence or absence of heteroduplexes. The detection is of a heteroduplex indicates the presence of a base pair mismatch and/or an insertion/deletion in the sample fragment(s). Although comparative sequencing can be carried out using any of a number of analytical methods, it is particularly suited to the denaturing high performance liquid chromatography method of the present invention.

Detailed Description Paragraph Right (15):
In the context of the present invention, in instances in which only homoduplexes are observed during the sample screening, further standard sequencing is not required since the sequence is monomorphic in all subjects compared. In utilizing the method of the present invention, only those DNA fragments identified as heteroduplexes, and therefore identified as containing at least one polymorphic site, are sequenced by conventional methods to characterize the observed polymorphism(s).

Detailed Description Paragraph Right (18):
The present invention provides a method for separating heteroduplex and homoduplex DNA molecules in a mixture using high performance liquid chromatography and more particularly, denaturing high performance liquid chromatography, as will be described in detail below. The method can be utilized for detecting a single base mismatch in a DNA duplex containing up to about 2000 base pairs.

Detailed Description Paragraph Right (19):
High performance liquid chromatography (HPLC) generally refers to a technique for partitioning a sample or more specifically the components of a sample between a liquid moving or mobile phase and a solid stationary phase. In the present invention, the applicants have discovered a chromatographic method which utilizes conditions effective for at least partially denaturing heteroduplexes during sample elution to thereby enable the separation and identification of heteroduplexes and homoduplexes contained in a mixture.

Detailed Description Paragraph Right (20):
In the method of the present invention, a sample mixture containing both heteroduplex and homoduplex molecules is applied to a stationary phase. Generally, the stationary phase is a reverse phase material in which the chemically bonded phase is hydrophobic and is less polar than the starting mobile phase. Any of a number of commercially available reverse phase solid supports may be utilized in the present nucleic acid separation method although the resolution may vary depending upon the nature of the sample and other relevant experimental parameters.

Detailed Description Paragraph Right (28):

For achieving separations of samples containing heteroduplexes and homoduplexes of up to about 2000 base pairs in size, the stationary phase will typically have a surface area of about 2-400 m.sup.2 /g, and preferably about 8-20 m.sup.2 /g as determined by nitrogen adsorption.

Detailed Description Paragraph Right (36):
In an alternate embodiment, the pH of the mobile phase is adjusted to effect at least partial denaturation of the heteroduplex molecules in a sample containing a mixture of homoduplexes and heteroduplexes to allow separation and detection of the heteroduplex molecules. In using chemical means to effect heteroduplex denaturation, the pH may be adjusted by addition of either base (e.g., sodium hydroxide or urea to a pH of around about 8) or acid (e.g., triethylamine and acetic acid at a pH of about 8) under conditions effective to at least partially denature the heteroduplex molecules and which do not degrade the nucleic acids present in the sample nor adversely affect the integrity of the stationary phase. In such cases, sample elution may be carried out at temperatures less than about 50.degree. C.

Detailed Description Paragraph Right (39):
One way to achieve the denaturing HPLC conditions of the present invention (e.g., effective to at least partially denature heteroduplexes) is to modulate column temperature, as will be discussed in reference to the Examples below.

Detailed Description Paragraph Right (40):
A column temperature typically between about 50.degree.-65.degree. C. is preferred for resolving heteroduplex molecules from their corresponding homoduplex molecules by denaturing HPLC chromatography. The optimal column temperature will depend upon the sequence (base composition) of the sample to be separated, the choice of stationary phase, the choice of mobile phase, pH, flow rate, and the like, and in many cases, will be determined empirically. Ideally, in cases with known sequence, a suitable column temperature may be calculated.

Detailed Description Paragraph Right (41):
As will be seen from the discussion of the Examples below, heteroduplex detection can, in many cases, be accomplished at a column temperature of about 56.degree. C. Column temperatures as low as about 50.degree. C. can be used for detecting point mutations in small nucleic acid fragments containing up to about 70 base pairs. Column temperatures up to about 63.degree. C. may be optimal for separating G-C rich fragments.

Detailed Description Paragraph Right (42):
In addition to providing a denaturing HPLC method for detecting heteroduplex molecules in a sample containing both heteroduplex and homoduplex molecules of essentially the same size, the present method can also be used to detect homoduplex molecules containing short single-stranded overhangs, as illustrated in Example 2. Further, by varying the column temperature, both partial and complete denaturation of a 30-mer homoduplex containing a 2-base overhang was detectable in the resulting chromatograms.

Detailed Description Paragraph Right (45):
In contrast, elution at 50.degree. C. was effective to denature all of the D-E hybrid molecules in sample, as seen in FIG. 2C. The two peaks in the resulting chromatogram correspond to single stranded oligonucleotides D and E, respectively. The above exemplary results demonstrate that under the denaturing HPLC conditions of the present invention, complementary hybrid molecules containing short overhangs can be denatured and that the extent (e.g., percentage of molecules denatured) of denaturation is a function of temperature. Utilizing the denaturing conditions of the present invention, short homoduplex fragments (i.e., less than about 70 base pairs in length) carrying short overhangs may be detected due to their denaturation during sample elution and detected in the resulting chromatograms as their corresponding single-stranded components.

Detailed Description Paragraph Right (46):
In initial experiments to test the general applicability of the method, a short heteroduplex 43-mer containing a single base mismatch and lacking an overhang was eluted using the denaturing conditions of the present invention to investigate whether

or not such conditions would be effective in denaturing the heteroduplex. The details of the experiment are described in Example 3.

Detailed Description Paragraph Right (47):
Briefly, liquid hybridization of two 43-mers, oligonucleotide C and oligonucleotide A, a 43-mer complementary, with the exception of one base located ten bases from the 5' end of oligo A, to oligonucleotide C, was carried out by heating an equimolar mixture of the two subject oligonucleotides. The resulting A-C heteroduplex containing a single base pair mismatch was then chromatographed on a C18-polystyrene-divinylbenzene support using a binary gradient system composed of 0.1 TEAA (triethylammonium acetate) and 0.1 TEAA containing 25% acetonitrile at two different temperatures, 40.degree. C. and 51.degree. C., respectively. Details of the method including the gradient profile used to elute the sample are provided in Example 3.

Detailed Description Paragraph Right (48):
The results of the above described chromatography are shown in FIGS. 3A-C and 4A-C. In contrast to the results described above for the 30-mer homoduplex with a 2-base overhang, at the lower operating temperature of 40.degree. C. (FIGS. 3A-C), elution of the A-C hybrid resulted in a chromatogram corresponding to the annealed oligonucleotide fragments (heteroduplex A-C), as indicated in the resulting chromatogram by a single peak with a retention time differing from that of either oligo A or C. Upon raising the column temperature to 51.degree. C., complete denaturation of the sample was observed, as indicated in FIGS. 4A-C by the detection of two single peaks with retention times corresponding to those of single stranded oligonucleotide A and single stranded oligonucleotide C, respectively. In the exemplary chromatography described above, the effect of increasing column temperature on denaturation and subsequent detection of a heteroduplex molecule under a given set of chromatographic conditions is shown. The above results also demonstrate the ability of the method to discriminate small heteroduplex molecules in a sample by denaturing HPLC and indicate a beneficial feature of the method, namely, to separate oligonucleotides not only as a function of their size but also as a function of their respective sequence.

Detailed Description Paragraph Right (49):
In further support of the method of the present invention, experiments have been carried out in which single base mismatches in heteroduplexes to 1.5 kilobase in size have been detected, as will be described below. Further, a single set of chromatographic conditions was effective for detecting single base mismatches in mixtures of homoduplex and heteroduplex molecules from about 200-1500 base pairs in size, demonstrating the general applicability of the method.

Detailed Description Paragraph Right (50):
In an exemplary method for detecting polymorphisms in nucleic acid fragments, experiments were carried out in which two different homoduplex molecules, identical in sequence with the exception of one base pair, were denatured and reannealed to form a mixture containing four resulting duplex products, two homoduplexes and two heteroduplexes. Experiments were carried out with pairs of homoduplexes as described above containing 209, 439, 1000, and 1500 base pairs, respectively. Details of the experiments are described in corresponding Examples 4, 5, and 6A,B. A generalized scheme illustrating the mixture of products formed by denaturing and reannealing two such homoduplexes is provided in FIG. 5.

Detailed Description Paragraph Right (52):
Additional clonal types of all possible nucleotide substitutions at the polymorphic position, including 1, 2, 3, and 4 base pair insertion length variants were constructed using oligonucleotide cassette site-specific mutagenesis techniques. Briefly, short synthetic double stranded oligonucleotide inserts containing the desired nucleotide composition were subcloned into the unique Hpa I and Bg1 II restriction sites which flank the polymorphic site. Each of the clonal allelic states was confirmed by conventional DNA sequencing. These clones provided precisely defined reagents for subsequent heteroduplex formation and detection. Desired polynucleotide fragments of variable length up to 1500 base pairs, each containing a single known polymorphic nucleotide, were generated by amplifying fragments from the appropriate plasmid clone using sets of PCR primers complementary to the vector sequence which flank the polymorphic locus at various defined distances.

Detailed Description Paragraph Right (54):
Double-stranded DNA homoduplex A, "homo-A-209", a 209-base pair fragment, was composed of two complementary 209-base fragments, polynucleotides 1 and 2. Double stranded DNA homoduplex G, "homo-G-209", a second 209-base pair fragment, was identical in sequence to homo-A-209 with the exception of one base pair (a G-C substituted for A-T present in homo-A-209) and was composed of polynucleotides 3 and 4. Polynucleotide 3 was identical in sequence to polynucleotide 1, with the exception of a guanosine at position 168 from the 5' end of polynucleotide 3, in comparison to an adenosine at the analogous position in polynucleotide 1. In a similar fashion, polynucleotide 4 was identical in sequence to polynucleotide 2, with the exception of a cytosine at position 42 from the 5' end replacing a thymidine in the same position in polynucleotide 2.

Detailed Description Paragraph Right (55):
The resulting mixture of products, containing original homoduplexes homo-A-209 and homo-G-209 and newly formed heteroduplexes hetero-AC-209 and hetero-GT-209 were then analyzed under the denaturing conditions of the present invention using ion pairing reverse phase HPLC. The designation hetero-AC-209 represents the double stranded product formed by annealing polynucleotides 1 and 4, and contains a single base pair A-C mismatch at position 168 relative to oligo 1. The designation hetero-GT-209 represents the double stranded product formed by annealing polynucleotides 2 and 3, and contains a single base pair G-T mismatch at position 168 relative to polynucleotide 3.

Detailed Description Paragraph Right (57):
At a less stringent column temperature of 50.degree. C. (FIG. 6C), separation/detection of the individual components of the product mixture containing homo-A-209, homo-G-209, hetero AC-209, and hetero-GT-209 was not achieved (FIG. 6C). As seen in FIG. 6C, the components of the mixture co-eluted as a single peak, with a retention time about equivalent to that of each of homoduplexes homo-A-209 and homo-G-209 (FIGS. 6A,B).

Detailed Description Paragraph Right (58):
Upon raising the column temperature to 54.degree. C., the resolution of the separation was significantly enhanced as is shown in FIG. 7C. The two same-size homoduplex products were clearly separated both from each other and from the heteroduplexes, which eluted from the column slightly earlier than did the homoduplexes.

Detailed Description Paragraph Right (59):
Further to this point, FIGS. 10A-10C illustrate the sensitivity and resolving power of the method as a function of column temperature for samples of homo-A-209 and homo-G-209, subjected to denaturation and reannealing conditions, to produce a mixture containing homo-A-209, homo-G-209, hetero-AC-209, and hetero-GT-209. As seen in FIGS. 10A-C, at 50.degree. C. the mixture elutes as a single peak, with resolution improving at a heightened column temperature of 52.degree. C., and resulting in base line separation of the heteroduplexes from the homoduplex products at an optimized column temperature of 54.degree. C.

Detailed Description Paragraph Right (60):
In contrast to shorter double stranded DNA fragments having less than about 70 base pairs and containing a single base pair mismatch (e.g., Example 3), based on the number of peaks observed in FIG. 7C, larger DNA fragments appear to be only partially denatured using the optimized denaturing chromatographic conditions of the present invention, resulting in the formation of a "bubble" at the site of the base-pair mismatch. The distortion of the DNA duplex caused by this partial denaturation or bubble appears to cause a shift towards shorter retention times and allows the separation of heteroduplexes containing a single base pair mismatch from homoduplexes of the same size, as illustrated in FIG. 7C.

Detailed Description Paragraph Right (61):
The results described above show the effective separation of larger nucleic acid duplexes (i.e., over about 70 base pairs) containing a single base pair mismatch from homoduplexes of the same size by partial denaturation of the heteroduplexes using the denaturing HPLC conditions described herein, leading to shorter retention times.

Detailed Description Paragraph Right (63):
Summarizing the results, at a column temperature of 50.degree. C., each of the mixtures of homo- and heteroduplex products co-eluted and were detected as a single peak. At the lower column temperature, separation of the components of each of the reaction mixtures was not achieved, indicating the sensitivity of the separation method to temperature under the conditions employed.

Detailed Description Paragraph Right (64):
From the chromatograms shown in FIGS. 8C and 9C (column temperature of 56.degree. C.), it can be seen that the present method can be used for detecting single base pair mismatches in heteroduplexes up to at least 1 kilobase in size under chromatographic conditions similar to those used in the separation of shorter nucleic acid fragments, suggesting the general applicability of the method. Using the methods described herein, a single base pair mismatch was detected in polynucleotides 1500 base pairs in length, as illustrated in FIGS. 12A-12C. The results further suggest the potential for detecting single base pair mismatches in duplexes up to 2 kilobase in size.

Detailed Description Paragraph Right (65):
The method of the present invention can be used to detect heteroduplex molecules obtained by reverse transcription prior to amplification by PCR. The method of the present invention can also be extended to the quantification of competitive reverse transcriptase PCR (RT PCR) reactions, namely via the detection of competitive reverse formation. In competitive RT-PCR, heteroduplex formation is one potential consequence of the homology between competitor and native products. The identification of heteroduplex RT-PCR products can be an important factor in determining the quantitative accuracy of the competitive assay results.

Detailed Description Paragraph Right (66):
Generally, in competitive RT-PCR, an RNA homolog (competitor) which contains the same sequence which is recognized by the reaction primers for the RNA of interest (native) is modified either by changing the presence of a restriction site or by altering the length of the sequence intervening between the primer binding sites to provide competitor mutant RNA. A range of known quantities of competitor RNA is added to multiple reactions each containing uniform amounts of the RNA preparation to be quantified (native). DNA expression is estimated by observing the relative amount of native and competitor products resulting from RT-PCR (Ferre, et al.; Volkenandt, et al.). The production of heteroduplexes during RT-PCR amplification can be confirmed using the denaturing HPLC method of the present invention.

Detailed Description Paragraph Right (69):
In utilizing such an approach, the presence of a peak(s) in the resulting chromatogram that does not correspond to a product observed by gel electrophoresis may correspond to a heteroduplex.

Detailed Description Paragraph Right (70):
To confirm whether or not a peak detected by denaturing HPLC corresponds to a heteroduplex, native and competitor RNA are reverse transcribed and amplified in separate reactions. The amplification products are then mixed together, followed by IP-RP-HPLC analysis of this mixture. The mixture is then heated (e.g., at 97.degree. C. for 3 minutes) followed by cooling to (e.g., to 4.degree. C.), followed by a second IP-RP-HPLC analysis. The appearance of a new product peak, and comparison of the retention times of any new peaks to those of any unaccounted peaks observed in the chromatograms of the initial set of RT-PCR reactions, indicates heteroduplex formation.

Detailed Description Paragraph Right (71):
In addition to identifying single base pair mismatches, the HPLC method of the invention can also be used for detecting indels in nucleic acid fragments. In exemplary experiments similar to those described above, a 440-mer homoduplex was mixed with a 439-mer homoduplex, identical to the 440-mer, with the exception of a single base pair deletion. Following denaturation and reannealing, the resulting mixture, containing both the original homoduplex molecules and the newly formed heteroduplexes, was chromatographed by the denaturing HPLC method of the invention (pH 7.0, 55.5.degree. C., 0.1M TEAA/0.1M TEAA in 25% acetonitrile) and heteroduplex formation

was observed. The heteroduplex indel molecules were separated from the homoduplex starting reagents in the resulting chromatogram and exhibited shorter retention times than those of the corresponding 439-mer and 440-mer homoduplexes. Using the present method, similar results have been obtained for indels having two, three, and four base pair deletions.

Detailed Description Paragraph Right (76):
In one embodiment, the mobile phase components are introduced into a mixer inside the column oven and mixed prior to contact with the sample. Alternatively, the mobile phase components may be mixed at ambient temperature and contacted with the sample injector, also maintained at ambient temperature outside of the column oven. Both of the above variations have been shown to be suitable for detection of heteroduplexes as has been described.

Detailed Description Paragraph Right (79):
In investigating the effect of column length and pre-column equilibration on the detection of 1000-mer heteroduplexes, heteroduplex formation was detected using a 60 cm length of tubing between the injector and the column, with a total length of 50 cm maintained at a column temperature of 56.degree. C. Upon increasing the length of tubing to 100 cm (with a total length of 90 cm maintained at an oven temperature of 56.degree. C.), improved separation of the heteroduplex species was observed.

Detailed Description Paragraph Right (80):
As has been discussed in section IIB above, one parameter which impacts the denaturing HPLC method of the present invention is pH. Generally, the pH of the mobile phase is maintained between about 7 and 9. In attempting to observe a single base mismatch in polynucleotides 1.5 kb in length, a preferred pH for carrying out the separation was found to be 7.5, as is described in Example 6B and further illustrated in FIGS. 12A-12C, and particularly in FIG. 12 C, which indicates heteroduplex detection in polynucleotide molecules containing up to 1500 base pairs.

Detailed Description Paragraph Right (82):
In investigating the effect of sample sequence on the temperatures effective for heteroduplex separation, the denaturing HPLC method of the invention was used to detect polymorphic sites contained within the 31-A-G fragment of the .beta.-globin gene (Myers, et al., 1986), where the region containing the polymorphism is flanked by GC-rich segments.

Detailed Description Paragraph Right (85):
The results of the above experiment indicate that although in most cases a temperature of 56.degree. C. is effective for heteroduplex separation, in some instances, and particularly for polynucleotides containing polymorphisms flanked by GC-rich regions, higher temperatures may be necessary.

Detailed Description Paragraph Right (89):
Exemplary chromatograms of DYS199 samples are shown in FIGS. 11A and 11B, illustrating HPLC traces of (i) a sample containing only homoduplex molecules derived from samples of African and Italian origin (FIG. 11A), and (ii) samples of African and native South American origin, indicating the presence of heteroduplexes. Of the samples examined, only samples of native South American origin exhibited the detected polymorphism.

Detailed Description Paragraph Right (93):
The heteroduplex separation and detection method of the present invention based on heteroduplex formation (e.g., of PCR products) is faster, simpler, more sensitive and more informative than the currently available procedures (such as RNase A cleavage mismatch). The denaturing HPLC method of the invention detects heteroduplex molecules in a mixture containing both heteroduplexes and homoduplexes by utilizing conditions effective to at least partially denature the heteroduplexes. Under such denaturing conditions, heteroduplexes exhibit slightly different retention times (typically shorter) from their homoduplex counterparts, thus providing a sensitive and convenient assay for detecting heteroduplex formation.

Detailed Description Paragraph Right (94):
Using the conditions described above, base pair mismatches and indels can be observed in heteroduplexes using the method of the present invention. The preferred size range

for these heteroduplexes ranges from approximately 30 to 1000 base pairs in length, although larger-sized heteroduplexes can be used as well. Heteroduplexes formed from molecules with mismatched nucleotides were detected in duplexes having a degree of divergence was as low as about 0.1%, and even lower. In addition, more complex mixtures of restriction fragments (e.g., 100-1000 bp size range) resulting from the post-PCR digestion of longer amplification products can be surveyed for the presence of heteroduplexes.

Detailed Description Paragraph Right (101):
The present invention provides a method for comparative DNA sequencing in which potentially all possible nucleotide mismatches and insertion/deletions within select amplified DNA fragments obtained from multiple animal or human subjects can be detected. In the context of the present invention, comparative DNA sequencing is carried out by amplifying DNA samples, typically up to at least about 1.5 kb in length, obtained from multiple subjects. The amplified DNA fragments are then surveyed, either individually or in pools containing up to about 10 samples, for the presence or absence of heteroduplexes using the denaturing high performance liquid chromatography method of the present invention. In surveying the samples, the amplified DNA fragments are denatured and allowed to reanneal. The resulting mixture of DNA fragments is then applied to a stationary reverse-phase support. The sample mixture is eluted with a mobile phase containing an ion-pairing reagent and an organic solvent. Sample elution is carried out under conditions effective to at least partially denature any heteroduplexes present in the sample and results in the detection of any heteroduplex molecules contained in the sample. The detection of a heteroduplex indicates the presence of a base pair mismatch and/or an insertion/deletion in the sample fragment(s).

Detailed Description Paragraph Right (102):
In instances in which only homoduplexes are observed during the sample screening, further standard sequencing is not required since the sequence is monomorphic (e.g., lacking polymorphic sites) in all subjects compared. In utilizing the method of the present invention, only those DNA fragments identified as heteroduplexes, and therefore identified as containing at least one polymorphic site, are then sequenced by conventional methods to characterize the observed polymorphism(s).

Detailed Description Paragraph Right (106):
Typically, probes for any target nucleic acid can be selected from a region of the microorganism's genomic material, such as rRNA (for example, as in Weisburg, et al.). In this way probes can be identified that will form homoduplexes to identify specific species. Formation of heteroduplexes indicates that the sequences have diverged from the probe sequence.

Detailed Description Paragraph Right (115):
Another embodiment of the present invention is the use of specific probes to identify variants based on the formation of homoduplex complexes. For example, sequences corresponding to a particular virus variant can be cloned and amplified. These cloned sequences are then used as a probe against viral molecules isolated from a number of test sources. Using the method of the present invention, if homoduplexes are formed in hybridization reactions between the probe and the test source, then the test source is shown to be similar to the cloned probe variant. If on the other hand heteroduplexes are formed between the probe and test sequences, then sequence divergence between the probe and test sequences is indicated.

Detailed Description Paragraph Right (116):
With respect to cancer, once a diagnosis has been made, and a region of DNA associated with the cancerous growth has been identified, the heteroduplex separation method of the present invention can be used to evaluate the extent of infiltration of tumor cells within a tissue population. Exemplary potential target sequences are protooncogenes, for example, including but not limited to the following: c-myc, c-myb, c-fos, c-kit, ras, and BCR/ABL (e.g., Gazdar, et al.; Wickstrom; Zalewski, et al.; Calabretta, et al., 1992, 1993;), oncogenes/tumor suppressor genes (e.g., p53, Bayever, et al.). In tumor cells, deletions, insertions, rearrangements and divergent sequences in such genes or in the regions of DNA surrounding the coding sequences of such genes, all allow formation of heteroduplexes between amplified variant DNA and amplified DNA from normal cells.

Detailed Description Paragraph Right (118):
Typically, samples to be analyzed by the method of the present invention are obtained by polymerase chain reaction amplification--the amplified sequences are denatured and reannealed before HPLC analysis. In addition to obtaining nucleic acid samples by amplification, other samples sources can be used as well. For example, sequences of interest can be cloned (e.g., in a lambda vector; Sambrook, et al.) from two different sources. The sequences of interest are independently isolated away from vector sequences (e.g., by restriction endonuclease digestion and fragment purification). These two samples can then be combined, denatured, renatured, and the resulting heteroduplexes analyzed in accordance with the present method.

Detailed Description Paragraph Right (145):
These results demonstrate the effect of increasing column temperature on the denaturation of short oligonucleotide homoduplexes with single stranded overhangs and further illustrate that such species (e.g., homoduplexes containing base overhangs) are detectable even under partially denaturing conditions (e.g., 40.degree. C.).

Detailed Description Paragraph Right (155):
Table 1 shows all possible nucleotide substitution heteroduplex mismatches formed from appropriate plasmid clones and subsequently detected by denaturing high performance liquid chromatography for exemplary size fragments up to 1.5 kb in length.

Detailed Description Paragraph Right (157):
Double-stranded DNA homoduplex A, "homo-A-209", a 209-base pair fragment was composed of two complementary 209-base fragments, polynucleotide 1 (SEQ ID NO:5) and polynucleotide 2 (SEQ ID NO:6). Double stranded DNA homoduplex G, "homo-G-209", a 209-base pair fragment identical in sequence to homo-A-209 with the exception of one base pair (a G-C substituted for A-T present in homo-A-209) was composed of polynucleotide 3 (SEQ ID NO:7) and complementary polynucleotide 4 (SEQ ID NO:8). Polynucleotide 3 was identical in sequence to polynucleotide 1, with the exception of a guanosine at position 168 from the 5' end of polynucleotide 3, in comparison to an adenosine at the analogous position in polynucleotide 1. In a similar fashion, polynucleotide 4 was identical in sequence to polynucleotide 2, with the exception of a cytosine at position 42 from the 5' end replacing a thymidine in the same position in polynucleotide 2.

Detailed Description Paragraph Right (158):
The double stranded oligonucleotides homo-A-209 and homo-G-209 were subjected to denaturation and reannealing under the following conditions in a Perkin Elmer 9600 thermal cycler: 95.degree. C. for 3 minutes, followed by cooling from 95.degree. C. to 65.degree. C. at a rate of 1.degree. C. per minute (e.g., over a period of 30 minutes), followed by sample storage at 6.degree. C. A schematic representation of the products formed by denaturing the above 209-mer homoduplexes followed by reannealing is provided in FIG. 5. The resulting mixture of products, containing original homoduplexes homo-A-209 and homo-G-209 and newly formed heteroduplexes hetero-AC-209 and hetero-GT-209 were then analyzed by IP-RP-HPLC. Hetero-AC-209 represents the double stranded product formed by annealing oligonucleotides 1 and 4, and contains a single base pair A-C mismatch at position 168 relative to oligo 1. Hetero-GT-209 represents the double stranded product formed by annealing oligonucleotides 2 and 3, and contains a single base pair G-T mismatch at position 168 relative to oligo 2.

Detailed Description Paragraph Right (159):
Samples of each of homo-A-209, homo-G-209, and the resulting heteroduplexes formed by denaturation and naturation of homo-A-209 and homo-G-209 were then directly chromatographed on alkylated nonporous PS-DVB particles (as described above) packed into a stainless steel column (NPC18M HPLC Biopolymer column, 50.times.4.6 mm I.D., Serasep, Inc., Santa Clara, Calif.). Eluent A consisted of 0.1M TEAA (triethylammonium acetate), pH 7.0 and Eluent B consisted of 0.1M TEAA, pH 7.0, in 25 % acetonitrile. The samples were eluted using a linear gradient profile of 37-63% Eluent B in 5.5 minutes, followed by 90% Eluent B for 1.5 minutes, at a flow rate of 1 ml per minute (UV detection, 254 nm). Two separate runs were performed at 50.degree. C. and 54.degree. C. to optimize the effect of column temperature on separation of the product mixture components, as shown in FIGS. 6A-6C and FIGS. 7A-7C, respectively.

Detailed Description Paragraph Right (160):
At a less stringent column temperature of 50.degree. C., separation/detection of the product mixture containing homo-A-209, homo-G-209, hetero AC-209, and hetero-GT-209 was not achieved (FIG. 6C). However, upon raising the column temperature to 54.degree. C., the two homoduplex products were clearly separated from the heteroduplexes, which eluted from the column slightly faster than did the homoduplexes.

Detailed Description Paragraph Right (161):
In contrast to shorter double stranded DNA fragments having less than about 70 base pairs and containing a single base pair mismatch (e.g., Example 3), larger DNA fragments are only partially denatured using the optimized chromatographic conditions of the present invention, resulting in the formation of a "bubble" at the site of the base-pair mismatch. This partial denaturation or bubble causes a shift towards shorter retention times and allows the separation of heteroduplexes containing a single base pair mismatch from homoduplexes of the same size, as illustrated in FIG. 7C.

Detailed Description Paragraph Right (162):
Further to this point, FIGS. 10A-10C illustrate the sensitivity and resolving power of the method as a function of column temperature for samples of homo-A-209 and homo-G-209, subjected to denaturation and reannealing conditions, to produce a mixture containing homo-A-209, homo-G-209, hetero-AC-209, and hetero-GT-209. As seen in FIGS. 10A-C, at 50.degree. C. the mixture elutes as a single peak, with resolution improving at a heightened column temperature of 52.degree. C., and resulting in base line separation of the heteroduplexes from the homoduplex products at an optimized column temperature of 56.degree. C.

Detailed Description Paragraph Right (163):
In summary, the above results show the effective separation of larger nucleic acid duplexes (e.g. over 200 base pairs) containing a single base pair mismatch from homoduplexes of about the same size by partial denaturation of the heteroduplexes using the denaturing HPLC conditions described herein, leading to shorter retention times.

Detailed Description Paragraph Right (164):
In order to examine the sensitivity of the HPLC detection method of the invention, an experiment identical to that described in Example 4 above was carried out with two different DNA homoduplexes each containing 439 base pairs.

Detailed Description Paragraph Right (166):
The 439-base pair homoduplex A, designated as homo-A-439, was obtained by annealing polynucleotide 5 with polynucleotide 6. A 439-base pair homoduplex G, designated as homo-G-439, was obtained by annealing polynucleotide 7 with polynucleotide 8. Denaturation and reannealing were carried out as described above, generating a mixture containing homo-A-439, homo-G-439, and two resulting heteroduplex species.

Detailed Description Paragraph Right (167):
The resulting IP-RP-HPLC chromatograms for both homo-A-439 and homo-G-439 as well as for the sample obtained from denaturing and subsequent reannealing of homo-A-439 and homo-G-439 are shown in FIGS. 8A-8C, respectively. The column, column packing, composition of Eluent A and Eluent B, linear gradient, as well as the flow rate and detector wavelength are the same as those employed in Example 4. The separation was carried out at a column temperature of 56.degree. C. As seen in FIG. 8C, detection of the 439-mer heteroduplexes present in a sample also containing two 439-mer homoduplexes is achieved under the conditions employed, as evidenced by the peaks having shorter retention times and indicated by an arrow.

Detailed Description Paragraph Right (168):
The samples were also chromatographed at a column temperature of 50.degree. C., however, separation of the components of the reaction mixture containing both heteroduplex and homoduplex products was not achieved at the lower column temperature (not shown), since the products co-elute as a single peak.

Detailed Description Paragraph Right (169):
These results further support the general applicability of the method in separating and detecting single base pair mismatches in heteroduplexes containing nearly 500 base

pairs and further demonstrate the effect of column temperature in effecting at least partial denaturation of heteroduplex molecules in a sample containing both heteroduplexes and homoduplexes, as suggested by peaks (corresponding to the heteroduplex) having shorter retention times.

Detailed Description Paragraph Right (170):
To further investigate the separation capabilities of the present method, an experiment similar to that described in Example 5 above was carried out with two different DNA homoduplexes each 1 kilobase in length.

Detailed Description Paragraph Right (172):
The 1000-base pair homoduplex A, designated as homo-A-1kb, was obtained by annealing polynucleotide 9 with polynucleotide 10. 1000-base pair homoduplex G, designated as homo-G-1kb, was obtained in a similar manner. Denaturation and reannealing were carried out as described above to produce a sample containing homoduplexes homo-A-1kb and homo-G-1kb as well as the two heteroduplexes, hetero-AC-1kb and hetero-GT-1kb.

Detailed Description Paragraph Right (173):
The resulting IP-RP-HPLC chromatograms for both homo-A-1kb and homo-G-1kb as well as for the sample obtained from denaturing and subsequent reannealing of homo-A-1kb and homo-G-1kb are shown in FIGS. 9A-9C, respectively. The column, column packing, and composition of Eluent A and Eluent B were the same as those employed in Examples 4 and 5. A linear gradient of 40%-70% B in 7 minutes, followed by an increase to 90% B in 1.5 minutes was used to elute the products. A flow rate of 1 ml/minutes and a detector wavelength of 256 nm was employed. The separation was carried out at a column temperature of 56.degree. C. and resulted in the detection of 1-kilobase heteroduplexes containing only a single base pair mismatch in a sample also containing 1-kb homoduplexes.

Detailed Description Paragraph Right (174):
As in the previous examples, the samples were also chromatographed at a column temperature of 50.degree. C., however, separation/detection of the 1-kilobase heteroduplexes in a sample also containing 1-kilobase homoduplex products was not achieved at the lower column temperature (not shown). At the lower column temperature, the mixture of homo- and heteroduplex products co-elute and are detected as a single peak.

Detailed Description Paragraph Right (175):
The above results demonstrate the utility of the method in detecting single base pair mismatches in heteroduplexes up to 1 kilobase in size under chromatographic conditions similar to those used in the separation of shorter nucleic acid fragments, suggesting the general applicability of the method. The results further suggest the potential for detecting single base pair mismatches in duplexes larger than 1 kilobase in size.

Detailed Description Paragraph Right (176):
In a similar fashion to Example 6A described above, 1.5 kilobase heteroduplex molecules containing a single base pair mismatch were separated using the denaturing HPLC method of the present invention.

Detailed Description Paragraph Right (177):
The 1.5 kb fragment was amplified essentially as described above using primers SEQ ID NO:24 (forward) and SEQ ID NO:25 (reverse). The 1.5 kb A form sequence of polynucleotide 11 was annealed with polynucleotide 12 to create a 1.5 kb homoduplex, homo-A-1.5 kb. A similar 1.5 kb G form sequence was also generated from the complementary polynucleotides 13 and 14, homo-G-1.5 kb. Following mixing, denaturing and reannealing the 1.5 kb homo A and G duplexes, the single base mismatch of the resulting heteroduplex molecules was at position 829 from the 5' end of polynucleotides 11 and 13.

Detailed Description Paragraph Right (178):
The separation was effected at a pH of 7.5. The elution was carried out using a Gynkotek low-pressure gradient HPLC system (Gynkotek, Germering, Germany) at a temperature of 57.degree. C. and a flow rate of 1 ml/minute. A binary gradient system was employed (with components as described above), using a linear gradient profile of 62-71% Eluent B in 5 minutes. The chromatograms are shown in FIGS. 12A-12C, with FIG.

12C illustrating separation of the 1.5 kb heteroduplex molecules from the corresponding homoduplexes. The results suggest the potential for detecting single base mismatches in duplexes up to 2 kilobase in size using the present method.

Detailed Description Paragraph Right (186):
Exemplary chromatograms are shown in FIGS. 11A and 11B, illustrating HPLC traces of (i) a sample containing only homoduplex molecules derived from samples of Italian origin (FIG. 11A), (ii) in comparison with samples of native South American origin, indicating the presence of heteroduplexes.

Detailed Description Paragraph Left (8):
B. Denaturation/Reannealing of 209-mer Homoduplexes Formation and Detection of 209-mer Heteroduplexes

Detailed Description Paragraph Left (9):
B. Denaturation/Reannealing of 1500-Base Pair Homoduplexes: Formation and Detection of 1500-Base Pair Heteroduplexes

Detailed Description Paragraph Center (5):
IV. HPLC-Promoted Partial Denaturation and Detection of Large Heteroduplexes

Detailed Description Paragraph Center (20):
Denaturation/Reannealing of 439-mer Homoduplexes Formation and Detection of 439-mer Heteroduplexes

Detailed Description Paragraph Center (22):
A. Denaturation/Reannealing of 1000-Base Pair Homoduplexes: Formation and Detection of 1000-Base Pair Heteroduplexes

Detailed Description Paragraph Table (1):
TABLE 1 DUPLEX SPECIES Clone # A/T (1) G/C (2) T/A (3) C/G (4) A/T (1) a/t A/C + G/T A/A + T/T A/G + C/T G/C (2) G/T + A/C g/c G/A + T/C G/G + C/C T/A (3) T/T + A/A T/C + G/A t/a T/G + C/A C/G (4) C/T + A/G C/C + G/G C/A + T/G c/g Lower case = homoduplexes Upper case = heteroduplexes

Other Reference Publication (8):
Oefner, P.J., et al., "High Resolution Liquid Chromatography of Fluorescent Dye-Labeled Nucleic Acids," Anal. Biochem. 223:39-46 (1994).

Other Reference Publication (13):
Nagamine et al, "A PCR Artifact: Generation of heteroduplexes", Am. J. Hum. Genet. 45:337-339, 1989.

Other Reference Publication (15):
Keen et al, "Rapid detection of single base mismatches as heteroduplexes on hydrolink gels", Trends Genet. 7(1):5, Jan. 1991.

CLAIMS:

1. A chromatographic method for separating heteroduplex and homoduplex DNA molecules in a mixture, comprising:

applying the mixture to a stationary reverse phase support,

eluting the heteroduplex and homoduplex molecules of said mixture with a mobile phase containing an ion-pairing reagent and an organic solvent, where said eluting is carried out under conditions effective to at least partially denature said heteroduplexes and where said eluting results in the separation of said heteroduplexes from said homoduplexes.

19. The method of claim 1, where prior to said applying step the DNA molecules are amplified using the polymerase chain reaction and the amplified DNA molecules denatured and renatured to form a mixture of heteroduplex and homoduplex DNA

molecules.

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☑ 1. 6265557. 09 May 97; 24 Jul 01. ABO histo-blood group O alleles of the baboon. Diamond; David, et al. 536/23.1; 435/320.1 536/23.2 536/23.5. C07H021/02 C07H021/04 C12N015/00.

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Term	Documents
ALLELE-SPECIFIC.DWPI,EPAB,JPAB,USPT.	1399
ALLELE-SPECIFICS	0
PROBE$1	0
PROBE.DWPI,EPAB,JPAB,USPT.	211714
PROBEA.DWPI,EPAB,JPAB,USPT.	3
PROBEB.DWPI,EPAB,JPAB,USPT.	1
PROBEC.DWPI,EPAB,JPAB,USPT.	3
PROBED.DWPI,EPAB,JPAB,USPT.	12019
PROBEE.DWPI,EPAB,JPAB,USPT.	4
PROBEF.DWPI,EPAB,JPAB,USPT.	1
PROBEG.DWPI,EPAB,JPAB,USPT.	1
(ALLELE-SPECIFIC NEAR5 PROBE$1 NEAR5 LABEL$2 NEAR5 FLUORESCENT).USPT,JPAB,EPAB,DWPI.	1

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L2: Entry 1 of 1 File: USPT Jul 24, 2001

DOCUMENT-IDENTIFIER: US 6265557 B1
TITLE: ABO histo-blood group O alleles of the baboon

Detailed Description Paragraph Right (64):
Allele-specific PNA probes are constructed and are labeled with fluorescent markers such that the A-specific probe is red, the B-specific probe is blue, and the probe specific to the major (A-like) O allele is yellow. These labeled probes are used to determine the genotype of a biological sample. Assignment of fluorescence color for probes is arbitrary and is provided herein for convenience. A variety of fluorescence labels is known to those of ordinary skill in the art.